Involvement of p38MAPK in Impaired Neutrophil Bactericidal Activity of Hemodialysis Patients

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The involvement of p38MAPK in impaired neutrophil bactericidal activity of hemodialysis patients

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Abstract

Background: Mortality from infections has been reported to be higher in hemodialysis (HD) patients. Although dysfunction of neutrophils against bacterial infection was reported in HD patients, the precise mechanism remains to be clarified. We therefore examined the impacts of neutrophil inflammatory signaling on bactericidal activity in HD patients.

Methods: Comprehensive analyses of intracellular signalings were performed in whole blood of HD patients and control using microarray system. To confirm the contribution of the signaling to bactericidal activity in neutrophils, we examined the phosphorylation, bacterial killing function, reactive oxygen species (ROS) production and myeloperoxidase (MPO) release in neutrophils against staphylococcus aureus (*S. aureus*).

Results: RNA microarray analysis showed the suppression of p38 mitogen activated protein kinase (MAPK) signaling in HD patients. Neutrophils in HD patients showed the impairment of bactericidal activity against *S. aureus* compared to healthy subjects. Phosphorylation rate of p38MAPK of neutrophils in response to *S. aureus* was lower in HD patients than healthy subjects. The levels of ROS produced by neutrophils after co-culture with *S. aureus* were lower in HD patients, on the other hand, there was no difference of MPO release between HD patients and healthy subjects. A selective pharmacological inhibitor of p38MAPK suppressed bacterial killing function as well as ROS production in neutrophils of healthy subjects.

Conclusions: Impairment of p38MAPK signaling pathway might contribute to the suppression of neutrophil bactericidal activity in HD patients through the less production of ROS.

Keywords

Infection, neutrophils, dialysis, p38MAPK, ROS

Introduction

Infection is the second cause of death following cardiovascular diseases in patients on hemodialysis (HD) [1, 2]. Especially, the mortality rate of HD patients due to sepsis has been reported to be higher in every age bracket [3]. Numerous risk factors predispose HD patients to infection. Vascular access was a major risk factor for infection and bacteremia, and the most common micro-organism was staphylococcus aureus (*S. aureus*) in HD patients [4]. HD patients with *S. aureus* bacteremia are known to be at notable risk for endocarditis, which is a life-threating infectious disease [5, 6]. Other potential risk factors for infection include high burden of coexisting illnesses, hypoalbuminemia [7, 8], immunosuppressive therapy [9], nephrotic syndrome [10], uremia, anemia, malnutrition [11] and change of susceptibility of bacteria to antibiotics [12]. However, the precise reasons why HD patients are susceptible to bacteremia remain to be clarified.

Host defense systems to protect from infection comprise mechanical barriers such as skin, and non-specific (innate)/specific (adaptive) immune systems [13, 14]. Neutrophils are thought to be responsible for the first-line non-specific defense systems against bacterial infection through their anti-bacterial activities [14, 15]. Neutrophils migrate to the site of bacterial infection along a chemotactic gradient, ingest the invading microorganisms by phagocytosis and kill them with proteolytic enzymes such as reactive oxidative species (ROS) and myeloperoxidase (MPO). All of these functions are regulated by various intracellular signaling pathways in neutrophils. Previous studies reported impairments of neutrophil antibacterial activities such as migration, bactericidal activity and ROS production in patients on HD [16-18]. The elucidation of mechanisms focusing on intracellular signaling underlying the impaired immune response in neutrophils of HD patients are required to establish therapeutic strategies to combat bacterial infections.

Mitogen-activated protein kinases (MAPKs) are major intracellular signal transduction factors, through which signals from environmental stimuli are transmitted to the nucleus

[19]. Thus far, at least three distinct groups of MAPKs have been identified; extracellular signal-regulated kinase (ERK), p38MAPK and c-Jun NH2-terminal kinase (JNK). MAPKs are phosphorylated in response to various stimuli such as growth factors, oxidative stresses and inflammatory cytokines [20-21]. Then, phosphorylated MAPKs contribute to the activation of nuclear transcription factors regulating the gene expression of various molecules. Thus far, it has been reported that exposure to viable *S. aureus* led to the activation of ERK and p38MAPK in neutrophils from healthy volunteers [22, 36], suggesting that these intracellular signaling pathways are involved in bacterial clearance by neutrophils.

These findings prompted us to examine the comprehensive analysis focusing on intracellular signaling pathways using neutrophils from HD patients to explore the mechanisms of susceptibility of HD patients to bacterial infections. This study showed that p38MAPK signaling was impaired in neutrophils isolated from HD patients, which were associated with lower killing activity of neutrophils against *S. aureus* through the less production of ROS.

Material and Methods

Patients and control subjects

First, RNA microarray analysis was performed to evaluate the genes related to intracellular signaling in whole blood. For this analysis, 12 patients were enrolled, which were 4 HD patients (HD group) and sex- and age-matched 8 hypertensive patients without renal dysfunction (eGFR>60ml/min; control group). The patient characteristics are summarized in Table 1. The numbers of white blood cells and neutrophils were not different between both groups. Next, neutrophil functions were analyzed in separate groups including 7 HD patients (HD group) and sex- and age-matched 10 healthy volunteers (control group). The patient characteristics are summarized in Table 2. The number of white blood cells was not different between both groups. Blood samples were collected upon entry to the study after informed consent was obtained. This study was approved by the ethics committee of Kanazawa University (IRB approval number 285). None of patients or healthy volunteers had diabetes mellitus, malignant neoplasm and bacterial infection.

Reagents

RPMI 1640 was from Thermo Fisher Science (Waltham, MA, USA), and 10% human serum of type AB were from Takara (Kusatsu, Japan). SB202190 was obtained from Sigma-Aldrich (St. Louis, MO, USA). DMSO as a vehicle of SB202190, LB medium and Agar were from Wako (Osaka, Japan).

RNA isolation, microarray hybridization and analysis

For RNA microarray analysis, RNA was isolated by using PAXgene Blood RNA System Kit (PreAnalytiX QIAGEN/BD, Hombrechtikon, Switzerland) as previously described [23,24]. In brief, blood specimens (2.5 ml) were collected in PAXgene tubes from each patient. RNA was extracted from whole blood following the manufacturer's protocol. Total RNA (100 ng) was used for the generation of biotin-labelled cRNA

using the Affymetrix Two-Cycle cDNA Synthesis Kit (Affymetrix, Santa Clara, CA). The biotin-labelled cRNA was hybridized to Affymetrix HG-U133 Plus2.0 GeneChips after fragmentation. After the chips were washed followed by a staining with streptavidin and phycoerythrin, they were scanned by using the GeneChip scanner 3000 (Affymetrix). Raw data file formats were generated using the Gene Chip Operating Software (Affimetrix). The gene expression analysis was performed using BRB ArrayTools ver 4.41 (NCI, http://linus.nci.nih.gov/BRB-ArrayTools.html). We performed pathway class comparison of Bio Carta database between the HD group and the control group. Only those categories with p value < 0.0001 for LS and KS permutation statistical tests were considered as statistically significant.

Neutrophil isolation

Neutrophils were isolated from heparinized whole blood (10 ml) that was collected from HD patients or healthy volunteers. The blood was layered over a Polymorphprep resolving media (Accurate Chemical, Westbury, NY) and spun at 500 x g for 35 min. The neutrophil rich fraction was collected, and red blood cells were lysed with BD PharmLyse (BD Biosciences, San Jose, CA). The number of neutrophils was counted by Cellometer mini (Nexcelom Bioscience, Lawrence, MA). The purity and the viability of neutrophils were assessed by Giemsa staining (Diff-Qik; Gentaur Europe, Brussels, Belgium) and flow cytometry analysis of lactoferrin (Beckman Coulter, Brea, CA), respectively. The purity and the viability of neutrophil preparations was over 95%. The neutrophils were washed twice with PBS and then resuspended in RPMI 1640 supplemented with 10% human serum of type AB without antibiotic-antimycotic reagent.

Bacterial killing assay

Bacterial killing assay was performed as described previously [25]. Briefly, *S. aureus* (strain Smith, kindly provided by Dr. Suda, Kanazawa University, Ishikawa, Japan) was grown in LB medium until OD 1.9–2.2 at 600 nm. After opsonized with 10% human serum of type AB for 30 min at 37 ºC in a water bath, the *S. aureus* and 1×10^5 neutrophils were incubated together at 1:1 ratio for 240 min at 37ºC 5% CO_2 on 96 wells plates. Then, neutrophils were lysed by adding distilled H_2O and diluted

aliquots were spread on LB agar plates. After the incubation overnight at 37℃, we counted the number of CFU at each plate and calculated the growth rate of *S. aureus* as the ratio of the number of CFU at 240 min to the number of CFU at baseline.

Western blot

1×10^6 neutrophils were co-cultured with either opsonized *S. aureus* at 1:1 ratio or stimulated by 10μM N-Formyl-Met-Leu-Phe (fMLP) for 5min (37℃, 5% CO2), and then immediately they were put on ice. After the cells were spun down at 3000rpm 4℃ for 5min, each pellets were lysed in 100 μl of lysis buffer (10 mM Tris–HCl (pH 7.4), 1% SDS). A total of 10 μg proteins were loaded per well and separated on a 7% SDS–PAGE by electrophoresis and then transfer to a nitrocellulose membrane by using iBlot 2 Gel Transfer Device (Thermo Fisher Science). The transferred nitrocellulose membranes were probed with anti-p38MAPK antibody (Cell Signaling Technology, Danvers, MA, USA) or anti-phosphorylated p38MAPK antibody (Cell Signaling Technology) at a concentration of 1:2000. The secondary goat anti-rabbit antibody conjugated with horseradish peroxidase was used at a concentration of 1:5000 (Cell Signaling Technology). The membrane was visualized by chemiluminescence using the ECL kit (Amersham Biosciences, Piscataway, NJ). Phosphorylation rate of p38MAPK was assessed from each protein expression level by using ChemiDoc Touch Imaging System (Bio-Rad Microscience, Hercules, CA, USA).

Measurements of ROS production and MPO activity

To examine ROS production and MPO activity in neutrophils, 1×10^5 neutrophils were measured after co-culture with *S. aureus* for 30min (37℃, 5% CO2). ROS production was measured by using an Oxi Select Intracellular ROS assay kit (Cell Biolabs, Inc., San Diego, CA, USA), which uses the oxidation-sensitive fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) rapidly oxidized to highly fluorescent 2', 7'- Dichlorodihydrofluorescein (DCF) by ROS. MPO activity was measured by using Neutrophil Myeloperoxidase Activity Assay Kit (Cayman Chemical Company, MI, USA). One unit of MPO is defined as the amount of MPO which generates taurine chloramine to consume 1.0 μmol of Trinitrobenzenesulfonic per

minute at 25 ℃. Fluorescence intensities were measured by plate reader (Tecan, Salzburg, Austria) at 480/530 nm for ROS assay or at 650/530 nm in MPO assay.

Lactate dehydrogenase (LDH) assay

LDH assay was performed to examine the viability of neutrophils using LDH cytotoxicity detection kit according to a manufacture's protocol (Takara Bio Inc. Kusatsu, Japan).

Statistical analysis

The results are expressed as mean values ±SEM. One-way analysis of variance (ANOVA) by the Bonferroni method or the Student's *t* test was used to determine the significance of differences between groups in this study. In all analyses, statistical significant was set at p value < 0.05.

Results

The gene expression profiles of p38MAPK Signaling were preferentially declined in hemodialysis group.

BioCarta pathway analysis revealed that 5 out of 264 investigated gene sets passed the $P<0.0001$ significance threshold in whole blood, such as MAPK Signaling Pathway and p38MAPK Signaling Pathway (Figure 1a). Therefore, the expression levels of genes associated with p38MAPK signaling pathway were examined as shown in Figure1b. As a result, 43 out of 48 p38MAPK signaling-associated genes were down-regulated (Figure 1b). ERK and JNK signaling pathway did not show any statistical significance.

Phosphorylation of p38MAPK was impaired in neutrophils of HD patients as compared to healthy subjects with or without bacterial stimuli.

To confirm the impairment of p38MAPK signaling in neutrophils of HD group, we examined the phosphorylation of p38MAPK in neutrophils. As shown in Figure 2a-b, neutrophils of HD group demonstrated the suppression of p38MAPK phosphorylation as compared to neutrophils of control group without any stimuli. Next, we examined if bacterial stimulation is capable of inducing p38MAPK activation in neutrophils. After co-culture of neutrophils with *S. aureus* for five minutes, p38MAPK phosphorylation of neutrophils was much lower in HD group than in control group (Figure 2a-b). The bacterial chemotactic peptide fMLP has been reported to induce the phosphorylation of p38MAPK in neutrophils, therefore, it is thought to be able to modulate neutrophil functions [26]. As shown in Figure 2a-b, fMLP-induced phosphorylation of p38MAPK in neutrophils was also suppressed in HD group as compared to control group. We did not see any difference of neutrophil viability between both groups (Figure 2c). These results suggest that p38MAPK signaling was significantly impaired in HD patients-derived neutrophils both at baseline and after bacterial stimulation.

The bactericidal activity of neutrophils was suppressed in HD patients.

Neutrophils are the most abundant leukocytes in humans and essential to the innate

immune responses against bacterial invasion [15]. In addition, p38MAPK is known to be involved in multiple cellular processes such as cell growth, differentiation and cytokine productions [27]. Therefore, we hypothesized that the impairment of p38MAPK signaling contributes to the susceptibility of HD patients to bacterial infections. To evaluate the bactericidal activity of neutrophils against *S. aureus*, we performed bacterial killing assay by co-culturing neutrophils with *S. aureus*. As shown in Figure 3a, the growth rate of *S. aureus* showed about six-fold increase without neutrophils. However, the co-culture of *S. aureus* with neutrophils of control group inhibited the growth of *S. aureus* almost completely (130.0±49.6%). On the other hand, the growth rate of *S. aureus* was increased by 376±201.1% with neutrophils of HD group ($p<0.05$), indicating that the bactericidal activity of neutrophils was suppressed in HD group compared to control group. There was no difference in neutrophil viability between both groups at each time point of this assay (Figure 3b).

The levels of ROS production in neutrophils after co-culture with *S. aureus* were lower in HD patients than those in healthy subjects.

Neutrophils are capable of generating high amount of ROS by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase following phagocytosis to destroy invading bacteria [15]. To investigate whether neutrophil ROS production is dysregulated in HD patients resulting in the loss of bactericidal activity, we measured the levels of ROS by the stimulation with *S. aureus*. Under the control state, the levels of ROS production in neutrophils of HD group showed the declining trend as compared those in control group, although it was not statistically significant (Figure 4a). After co-culture with *S. aureus* or fMLP, the levels of ROS production were lower in neutrophils of HD group than control group, suggesting the involvement of ROS dysregulation in disturbed bactericidal ability of HD group (Figure 4a). In addition, MPO is a major component of neutrophilic granules and released from activated neutrophils, thereby playing critical roles in degrading invading pathogen [15]. We hypothesized that MPO release was also inhibited in HD group after the stimulation with S. aureus, however, there was no difference between both groups in neutrophil MPO activities after co-culture with *S. aureus* (Figure 4b). These findings suggest that low bactericidal activity of neutrophils in HD patients may be due to the disturbance of ROS production.

The selective pharmacological inhibitor of p38MAPK suppressed the bactericidal activity and the levels of ROS production in neutrophils of healthy subjects.

As the mechanisms of ROS production in neutrophils, p38MAPK signaling has been reported to play important roles through the regulation of NADPH oxidase [21]. To confirm the involvement of p38MAPK signaling in bactericidal activity in neutrophils, we examined the effect of the selective pharmacological p38MAPK inhibitor SB202190 on neutrophil bactericidal activity as well as ROS production. First, LDH assay did not show any cell toxicity of SB202190 (date not shown). As shown in Figure 5a, bacterial killing assay showed five-fold increase of the growth rate of *S. aureus* without neutrophils whereas co-culture of *S. aureus* with healthy neutrophils almost totally inhibited the growth of *S. aureus*. In contrast, treatment with the SB202190 significantly suppressed bacterial killing function of healthy neutrophils in a dose dependent manner (Figure 5a). In addition, we investigated the effect of the p38MAPK signaling on the production of ROS as an anti-bacterial mediator. In the healthy neutrophils, ROS production was induced by a co-culture with *S. aureus*, however, it was suppressed by the treatment with SB202190 (Figure 5b).

Discussion

In this study, we found that p38MAPK signaling pathway was impaired in whole blood, especially in neutrophils of HD patients. Comprehensive analyses of intracellular signalings in neutrophils using microarray system showed the down-regulation of p38MAPK signaling-related genes, and phosphorylation of p38MAPK was also suppressed in HD patients under control state in western blotting. The extent of p38MAPK phosphorylation by co-culturing neutrophils with *S. aureus* was also lower in HD patients than in healthy subjects. Bacterial killing assay to examine the neutrophil bactericidal activity showed that *S. aureus* growth rate was higher in HD patients than that in healthy subjects. In addition, the levels of ROS from neutrophils after co-culture with *S. aureus* were lower in HD patients than those in healthy subjects, suggesting that neutrophil bactericidal function was dysregulated in HD patients through the impairment of ROS production. Furthermore, the treatment with p38MAPK inhibitor suppressed bactericidal function as well as ROS production in neutrophils. From these findings, we conclude that the impaired p38MAPK signaling pathway might contribute to the suppression of neutrophil bactericidal activity in HD patients through the dysregulation of ROS production.

Neutrophils have been well-known to play essential roles for acute inflammation. Neutrophils are recruited to the sites of acute bacterial infection, and significantly contribute to the elimination of pathogens through various anti-bacterial functions. Typically, neutrophils phagocyte pathogens, and encapsulate them in phagosomes. Then, neutrophils kill encapsulated pathogens using NADPH oxygenase-dependent ROS production or release of antibacterial proteins such as MPO from granules [28]. In this study, neutrophils of HD patients failed to effectively kill *S. aureus*, and showed lower activation of p38MAPK signaling as well as less production of ROS as compared to neutrophils of healthy subjects. Pharmacological inhibition of p38MAPK significantly suppressed bacterial killing and ROS production even in healthy neutrophils. In contrast, the release of MPO by co-culture of neutrophils with *S. aureus* showed no difference in both group. These findings suggest that p38MAPK-ROS system is disturbed in neutrophils of HD patients, thereby reducing

bacterial killing activity in neutrophils. Previous study reported that neutrophil phagocytosis of *S. aureus* was not affected by p38MAPK inhibition [29], suggesting that phagocytosis may not be a major factor for p38MAPK-dependent bacterial killing. In addition, prior studies reported phagocytosis of neutrophils is not impaired by uremia [30, 31]. Further investigations will be required to clarify the role of phagocytosis in neutrophils of HD patients.

Clarifying the mechanisms through which neutrophils activate p38MAPK signaling leading to the clearance of *S. aureus* is important to discover therapeutic targets. Neutrophils are known to be activated by a diverse repertoire of receptors such as integrins, Fc-receptors, chemokine receptors and Toll-like receptors [15]. Among them, β_2-integrin (C11/CD18) has been reported to be critical in mediating neutrophil activation for host defense responses to bacterial infection [32]. CD18 deficiency in human causes leukocyte adhesion deficiency type I syndrome, in which these patients present early in life with repeated bacterial infections [33]. These phenotypes have also been reported to be recapitulated in CD18-deficient mice [34, 35]. As the signaling pathway downstream of β_2-integrin in neutrophils after engagement with *S.aureus*, spleen tyrosine kinase (Syk) has been reported to be a critical component [36, 37]. Syk-deficient neutrophils showed poor activation of the NADPH oxidase in response to *S aureus* through the impaired activation of p38MAPK. In addition, loss of Syk specifically in neutrophils was reported to result in reduced clearance of *S. aureus* after subcutaneous or intraperitoneal infection [38]. Based on these findings and the data we found in this study, it may be possible to speculate that β_2-integrin activation could be impaired in neutrophils of HD patients. Previous study in healthy dogs showed that the expression levels of CD11/CD18 on neutrophils was not changed by hemodialysis procedure itself [39]. Further studies will be needed to elucidate the precise mechanisms by which p38MAPK activation is impaired in neutrophils of HD patients.

Besides the roles of p38MAPK in host defense systems against bacterial infections, p38MAPK signaling has been linked to various inflammatory diseases including

rheumatoid arthritis, atherosclerosis, glomerulonephritis and diabetes [40-44]. p38MAPK signaling regulates the production of inflammatory cytokines and chemokines through the activation of transcriptional factors such as nuclear factor (NF)-kB, which in turn contributes to the signaling of inflammatory molecules via their receptors, thereby being recognized to be responsible for the vicious cycle of inflammations [45]. Therefore, p38MAPK has been extensively investigated to develop anti-inflammatory drugs in many inflammatory diseases [46-48]. However, recent study has shown that sepsis patients displayed a reduced capacity to activate NF-kB in inflammatory cells, suggesting that Sepsis-induced immunosuppression associates with a defect in the capacity to activate NF-kB [49]. In addition, mice lacking MAPK-activated protein kinase 2, which is a substrate of p38MAPK, showed diminished resistance to *L. monocytogenes* due to impaired control of bacterial growth [50]. From these findings and our data in this study suggest that p38MAPK inhibition should be done in appropriate time and place.

In summary, we have shown that p38MAPK signaling was impaired in neutrophils isolated from HD patients, thereby contributing to the lower killing activity of neutrophils against *S. aureus* through the less production of ROS. Given the fundamental involvement of these processes in the clearance of pathogens, our results suggest that p38MAPK signaling may be one of therapeutic targets for immunocompromised host taking hemodialysis.

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Conflict of Interest Statement

None declared.

Authors' Contributions

N.S., Y.K., and R.I. designed the research; Y.K. performed most of the experiments; N.S., T.M., A.S. and Y.S. performed the experiments; S.K., T.T., A.H., Y.I., M.S., K.F., R.I., T.S., S.K., and T.W. provided scientific advice; and N.S., Y.K., and T.W. wrote the manuscript. All authors reviewed the manuscript.

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Legends to figures

Figure 1. The gene expression profiles of p38MAPK Signaling were preferentially declined in hemodialysis (HD) group. (a) BioCarta pathway analysis (HD group; n = 4, control group; n = 8) by BRB-ArrayTools. 5 out of 264 investigated gene sets passed the $p < 0.0001$ vs controls. (b) The expression levels of genes associated with p38MAPK signaling pathway. The 43 out of 48 p38MAPK signaling-associated genes were down-regulated in HD group. MAPK; mitogen-activated protein kinase, EGF; epidermal growth factor, GPCRs; G protein-coupled receptors, ADP; adenosine diphosphate.

Figure 2. Phosphorylation of p38MAPK was suppressed in neutrophils of HD patients as compared to healthy subjects with or without bacterial stimuli. (a-b) The phosphorylation of p38MAPK in neutrophils by the stimulation with opsonized *S. aureus* or 10μM fMLP for 5 min. Quantification was performed with ChemiDoc Touch Imaging System and the data are expressed as mean intensities of phosphorylated p38MAPK bands relative to intensities of total p38MAPK bands ± SEM (n = 6/each group). (c) Neutrophil viability at each time point of this assay. Data are expressed as mean $\pm$ SEM (n = 6/each group). *$p < 0.05$. **$p < 0.05$ vs. control neutrophils at 0 min. #$p < 0.05$ vs. HD neutrophils at 0 min. NS; not significant.

Figure 3. The bactericidal activity of neutrophils was suppressed in HD patients as compared to healthy subjects. (a) The growth rates of *S. aureus* after the co-culture

with neutrophils from HD patients or controls. The growth rates of *S. aureus* were expressed as the ratio of the number of CFU at 240 min to the number of CFU at baseline ± SEM (n = 7/HD group and n = 10/control group). (b) Neutrophil viability at each time point of this assay (n = 6/each group). Data are expressed as mean ± SEM. *$p < 0.05$. NS; not significant.

Figure 4. The levels of ROS production in neutrophils after co-culture with *S. aureus* or fMLP were lower in HD patients than those in healthy subjects. (a) The levels of ROS production in neutrophils after the co-culture with *S. aureus* or fMLP for 30 min. Data are expressed mean ± SEM (n = 5/each group). (b) MPO activity in neutrophils after the co-culture with *S. aureus* for 30 min. Data are expressed mean ± SEM (n = 5/each group). $^{\dagger}p < 0.05$ vs. unstimulated control neutrophils. *$p < 0.05$. NS; not significant.

Figure 5. The selective pharmacological inhibitor of p38MAPK suppressed the bactericidal activity and the levels of ROS production in neutrophils of healthy subjects. (a) The effect of p38MAPK inhibition on the growth rates of *S. aureus* after the co-culture with neutrophils from healthy subjects. The growth rates of *S. aureus* were expressed as the ratio of the number of CFU at 240 min to the number of CFU at baseline ± SEM (n = 6/each group). (b) The levels of ROS production in neutrophils after the co-culture with *S. aureus* with or without p38MAPK inhibition. Data are expressed mean ± SEM (n = 6/each group). *$p < 0.05$.